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SPECIFIC RELEASE OF PLASMA MEMBRANE ENZYMES BY A PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

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Summary

The release of plasma membrane ecto-enzymes by a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* was investigated. There was no effect on L-leucyl-β-naphthylamidase, alkaline phosphodiesterase I and Ca^{2+}- or Mg^{2+}-ATPase, but substantial proportions of the alkaline phosphatase and 5′-nucleotidase were released. There was no simultaneous release of phospholipid and the solubilized enzymes were not excluded from Sepharose 6-B. It was therefore concluded that release was not a secondary consequence of membrane vesiculation but occurred as a result of the disruption of specific interactions involving the phosphatidylinositol molecule.

The phosphatidylinositol-specific phospholipases C from *Bacillus cereus* and *Staphylococcus aureus* have been shown to release alkaline phosphatase, a plasma membrane ecto-enzyme, from a wide range of tissues from several mammalian species [1—4]. One interpretation of these results was that molecules of alkaline phosphatase (EC 3.1.3.1), held in the membrane by specific interactions involving the phosphatidylinositol molecule, were released when this phospholipid was hydrolysed. An alternative interpretation, however, would be that membrane vesicles or other lipoprotein complexes of high molecular weight (carrying alkaline phosphatase) might be released from the membrane by the phospholipase C, perhaps as a result of diglyceride production [5, 6].

In the present communication we have attempted to distinguish between these two possibilities by looking for the release of additional membrane components induced by the phosphatidylinositol-specific phospholipase C from

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

S. aureus and by assessing the molecular weight of the released material.

Most materials and methods were as described in previous papers [4, 7] or are detailed in legends to tables. Chromatography of samples (1 ml) of supernatants from phospholipase C-treated tissues was carried out at 4°C on a column of Sepharose 6-B equilibrated in 0.1 M NaCl/50 mM Tris·HCl, pH 8.0. The column (33 x 2.6 cm) was eluted at a flow rate of 8 ml/h and 4-ml fractions were collected. The void volume (V_0), assessed with blue dextran, was 60 ml.

Supernatants from rabbit kidney microsomes, which had been treated with phosphatidylinositol-specific phospholipase C to release more than 70% of their alkaline phosphatase, showed no detectable increase in phospholipid content as compared with the control (less than 1% release). The alkaline phosphatase/lipid phosphorus ratio had increased from 1.5 in the microsomes to 150 μmol/min per μmol lipid phosphorus in the supernatant after phospholipase C treatment.

Supernatants from a wide range of phospholipase C-treated tissue preparations [4] and containing increased alkaline phosphatase were also examined for the release of two other plasma membrane ecto-enzymes, L-leucyl-β-naphthylamidase (EC 3.4.11.1) and alkaline phosphodiesterase I (EC 3.1.4.1). Little or no increase in the release of these enzymes was observed after phospholipase C treatment. Data for rat liver slices, homogenates and microsomes are shown in Table I. Relatively high control release of L-leucyl-β-naphthylamidase was observed from homogenates of some tissues and indicated the presence of a substantial proportion of soluble activity. The possibility that this soluble activity might have masked the release of a small proportion of plasma membrane activity cannot, therefore, be excluded in these

TABLE I

EFFECT OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ON RELEASE OF ENZYMES FROM RAT LIVER

Rat liver slices, homogenates or microsomes were incubated with phospholipase C (10 units/ml) as described previously [4]. The incubation mixtures were centrifuged at 150 000 x *g* for 40 min and the supernatants assayed for released alkaline phosphatase (EC 3.1.3.1) [4], alkaline phosphodiesterase I (EC 3.1.4.1) [8], L-leucyl-β-naphthylamidase (EC 3.4.11.1) [9] and 5′-nucleotidase (EC 3.1.3.5; for assay see legend to Table III). Values are means of duplicate determinations and are expressed as percentages of the activity determined at the same time in samples of unincubated homogenates (or microsomal suspensions). Values in parentheses represent percentages of initial activities recovered in samples following incubation (before centrifugation).

	Activity released (%)			
	Alkaline phosphatase	5′-Nucleotidase	L-Leucyl-β-naphthylamidase	Alkaline phosphodiesterase I
Tissue slices*				
Control	2.4	0.6	10.5	2.0
Phospholipase C-treated	42.0	13.5	10.1	2.0
Homogenates				
Control	4.8	1.3	30.4	3.4
Phospholipase C-treated	85.2	30.9	29.7	3.5
Microsomes				
Control	5.1 (116)	0.3 (94)	11.3 (91)	1.2 (99)
Phospholipase C-treated	104.0 (112)	88.3 (114)	16.3 (98)	1.4 (99)

*Mean of 4 experiments.

tissues. The further possibility, that the absence of these activities in the supernatants was due to their inactivation by the phospholipase C, was also investigated. However, as more than 90% of the original L-leucyl-β-naphthylamidase or alkaline phosphodiesterase activity was recovered from control or phospholipase C-treated microsomes (from rabbit kidney or rat liver) this possibility appeared unlikely. Supernatants from phospholipase C-treated lymphocytes were also assayed for Ca^{2+}- or Mg^{2+}-ATPase but, as Table II shows, little or no increase in the release of these activities was detected. During incubation (with or without phospholipase C) the total activity was reduced to 60—70% of its original value, but this should still have allowed detection of any release. Preliminary experiments with turkey erythrocytes [11] and rat mast cells (Batchelor, K., personal communication) suggested that the phospholipase C did not release the Ca^{2+}- or Mg^{2+}-ecto-ATPase [12, 13] from these cells either.

Many supernatants did, however, show an apparent increase in 5′-nucleotidase activity, but in some cases this could have been due to a non-specific hydrolysis of AMP by released alkaline phosphatase. In more detailed studies using rat liver (particularly rich in 5′-nucleotidase (EC 3.1.3.5)) and lymphocytes, β-glycerophosphate (a substrate for non-specific phosphomonoesterases but not for 5′-nucleotidase) was substituted for AMP in the assay. Under these conditions the supernatants showed little or no increase in phosphomonoesterase activity (Table III). Furthermore, as the levels of activity detected using this substrate were much smaller than those observed with AMP (Table III) it would appear that the observed hydrolysis of AMP could not have been due to a released, non-specific phosphomonoesterase. Confirmation of the release of 5′-nucleotidase from rat liver was obtained by using an adenosine deaminase-linked assay [16] in which non-specific phosphomonoesterases are saturated by the inclusion of 20 mM β-glycerophosphate in the as-

TABLE II

EFFECT OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ON RELEASE OF ENZYMES FROM LYMPHOCYTES

Pig lymphocytes [10] were washed and suspended (20%, v/v) in 0.29 M sucrose/10 mM glucose/10 mM NaCl/1 mM HEPES/NaOH, pH 7.4. 4.5 ml of this suspension was incubated with 0.5 ml of phospholipase C (final concentration, 10 units/ml) in 50 mM Tris · HCl, pH 7.4, for 90 min at 37°C. Incubation mixtures were then centrifuged and supernatants assayed for released enzyme activities as described in the legend to Table I. Values are expressed as percentages of the activity determined in unincubated lymphocyte suspensions. Values in parentheses represent percentages of initial activities recovered in samples following incubation (before centrifugation). ATPase (EC 3.6.1.3) activities were assayed in an incubation mixture containing (final volume 1 ml) 30 mM Tris · HCl, pH 7.4/5 mM ATP/$MgCl_2$ (4 mM) or $CaCl_2$ (4 mM) or EDTA (1 mM). Incubation was for 30 min at 37°C and P_i produced was assayed as described in the legend to Table III. ATPase activities were calculated from the difference between ATP hydrolysis in presence of Mg^{2+} or Ca^{2+} and of EDTA.

Enzyme	Activity released (%)	
	Control	Phospholipase C-treated
Alkaline phosphatase	1.6 (101)	121 (100)
5′-Nucleotidase*	3.3 (109)	161 (162)
Alkaline phosphodiesterase I**	0.51 (89)	0.78 (91)
Ca^{2+}-ATPase*	0.71 (66)	9.49 (66)
Mg^{2+}-ATPase*	0.61 (68)	0.92 (65)

*Mean of 3 experiments.
**Mean of 2 experiments.

TABLE III

SUBSTRATE SPECIFICITY OF PHOSPHOMONOESTERASE ACTIVITY RELEASED BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

Supernatants were prepared as described in legends to Tables I and II and assayed for phosphomonoesterase activity. The incubation medium (final volume 1 ml) was essentially as described before [15] and contained either 5 mM AMP or β-glycerophosphate. The reaction was stopped with 1 ml 40% w/v trichloroacetic acid, centrifuged and 1 ml samples of the supernatant assayed for P_i by the method of Baginski et al. [16]. Values are the means of duplicate incubations. Total activities of samples were determined before incubation.

Tissue	Substrate	Activity in supernatant (nmol/min per ml)		Total activity (nmol/min per ml)
		Control	Phospholipase C-treated	
Liver				
Slices*	AMP	8.6	165	1680
	β-Glycerophosphate	5.3	4.3	22
Microsomes	AMP	1.9	480	540
	β-Glycerophosphate	0.7	1.4	1.6
Lymphocytes**	AMP	0.7	40	26
	β-Glycerophosphate	1.9	2.7	4.7

*Mean of 2 experiments.
**Mean of 3 experiments.

say medium. The conclusion that AMP hydrolysis was due to 5′-nucleotidase was further strengthened by the observation that omission of the β-glycerophosphate from this assay only increased the activity by about 10%.

The proportion of the alkaline phosphatase activity released from rat liver slices by the phospholipase C appeared substantially greater than the proportion of 5′-nucleotidase which was released (Table I). In the case of lymphocytes, however, all of the 5′-nucleotidase could be released (Table II), thus suggesting that in rat liver the 5′-nucleotidase may be relatively inaccessible to the phospholipase C action. The increased release from rat liver homogenates and microsomes supports this conclusion.

To demonstrate that the released enzymes were truly soluble and not bound to high molecular weight lipoprotein complexes such as membrane vesicles, chromatography of supernatants from phospholipase C-treated tissue slices, homogenates and microsomes, was carried out on a Sepharose 6-B column. Both alkaline phosphatase and 5′-nucleotidase entered the gel and were eluted as single peaks. No activity was detected in the void volume. The amounts of activity recovered in the eluate were approximately 90 and 65% of applied activity for alkaline phosphatase and 5′-nucleotidase, respectively. Mean V_e/V_0 values (3 experiments each) were 2.05 and 1.98 for rabbit kidney alkaline phosphatase and rat liver 5′-nucleotidase, respectively. For alkaline phosphatase solubilized from rabbit kidney homogenates by butanol extraction [18] this value was 2.02 (three experiments). Lactate dehydrogenase (molecular weight, 140 000) was eluted in a similar position (V_e/V_0 = 2.07), which suggested that the molecular weights of the released enzymes were in the region 100 000—200 000.

The data presented in this paper provide substantial evidence against the release of alkaline phosphatase and 5′-nucleotidase as a secondary consequence of membrane vesiculation. First, the released enzymes were not excluded from Sepharose 6-B and were eluted with apparent molecular weights similar to

those reported for purified enzymes. Butanol solubilization has been widely used as the initial step in the purification of alkaline phosphatase from a range of tissues and molecular weight determinations, by gel chromatography or sedimentation, have usually indicated molecular weights in the range 100 000—200 000 [18—23]. Detergent-solubilized 5′-nucleotidase has been reported to have a molecular weight of 140 000—150 000 [24]. Secondly, the ratio of alkaline phosphatase to phospholipid in the supernatant was 100 times greater than in the microsomes and thirdly, there was no significant release of several other enzymes (L-leucyl-β-naphthylamidase, alkaline phosphodiesterase and the Ca^{2+}- or Mg^{2+}-ATPase) which have also been suggested to be located at the outer surface of the plasma membrane [12, 13, 25—29].

The release of 5′-nucleotidase activity by the action of the phosphatidylinositol-specific phospholipase C identifies this enzyme as another protein which appears to be held in the membrane by specific interactions involving the phosphatidylinositol molecule, and we have recently reported that acetylcholinesterase is associated with the outer surface of the erythrocyte membrane in a similar manner [11]. The argument for a specific interaction of these enzymes with phosphatidylinositol is based primarily on the observation that their release is effected by a phospholipase C preparation which hydrolyses phosphatidylinositol but causes no detectable hydrolysis of other membrane phospholipids [31]. In the case of alkaline phosphatase it has also been demonstrated that such specific release is not effected by phospholipases showing broader substrate specificities or by detergents, proteinases, lipase or neuraminidase (see ref. 30).

The nature of the interaction between these ectoenzymes and phosphatidylinositol is not yet known but it is perhaps relevant to note that a specific interaction between a membrane penicillinase and phosphatidylserine in *Bacillus licheniformis* has been demonstrated to involve covalent linkage with the phospholipid headgroup [31, 32].

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